Exempting Homologous Pseudogene Sequences from Polymerase Chain Reaction Amplification Allows Genomic Keratin 14 Hotspot Mutation Analysis

Petra H. L. Hut, Pieter v. d. Vlies, Marcel F. Jonkman,* Edwin Verlind, Hiroshi Shimizu,†
Charles H. C. M. Buys, and Hans Scheffer

Department of Medical Genetics, University of Groningen, Groningen, The Netherlands; *Department of Dermatology, University Hospital Groningen, Groningen, The Netherlands; †Department of Dermatology, Hokkaido University School of Medicine, Sapporo, Japan

In patients with the major forms of epidermolysis bullosa simplex, either of the keratin genes *KRT5* or *KRT14* is mutated. This causes a disturbance of the filament network resulting in skin fragility and blistering. For *KRT5*, a genomic mutation detection system has been described previously. Mutation detection of *KRT14* on a DNA level is, however, hampered by the presence of a highly homologous but nontranscribed *KRT14* pseudogene. Consequently, mutation detection in epidermolysis bullosa simplex has mostly been carried out on cDNA synthesized from *KRT5* and *KRT14* transcripts in mRNA isolated from skin biopsies. Here we present a genomic mutation detection system for exons 1, 4, and 6 of *KRT14* that encode the 1A, L1-2, and 2B domains of the keratin 14 protein containing the mutation hotspots. After cutting the *KRT14* pseudogene genomic sequences with restriction enzymes while leaving the homologous genomic sequences of the functional gene intact, only the mutation hotspot-containing exons of the functional *KRT14* gene are amplified. This is followed by direct sequencing of the polymerase chain reaction products. In this way, three novel mutations could be identified, Y415H, L419Q, and E422K, all located in the helix termination motif of the keratin 14 rod domain 2B, resulting in moderate, severe, and mild epidermolysis bullosa simplex phenotype, respectively. By obviating the need of *KRT14* cDNA synthesis from RNA isolated from skin biopsies, this approach substantially facilitates the detection of *KRT14* hotspot mutations. ***Key words: epidermolysis bullosa simplex/ KRT14. J Invest Dermatol 114:616–619, 2000***

Epidermolysis bullosa simplex (EBS) is characterized by intraepidermal skin blistering as a result of mild trauma. According to clinical criteria, EBS is classified into three major subgroups (Fine *et al*, 1991). The most severe form, EBS–Dowling–Meara (DM), is characterized by generalized blistering with a circinate configuration. At the electron microscopy level, EBS-DM is identifiable by the presence of large cytoplasmic clumps of tonofilaments in the basal keratinocytes (Anton and Schnyder, 1982; Niemi *et al*, 1983; McGrath *et al*, 1992). EBS–Koebner (K) and EBS–Weber–Cockayne (WC) are milder forms. In EBS-K blistering is generalized whereas in EBS-WC it is confined to the hands and feet (Haneke and Anton, 1982). In the majority of families, EBS is inherited as an autosomal dominant disorder. In rare cases, however, the disease is transmitted in an autosomal recessive mode [recessive epidermolysis bullosa simplex (REBS); Chan *et al*, 1994; Rugg *et al*, 1994; Jonkman *et al*, 1996].

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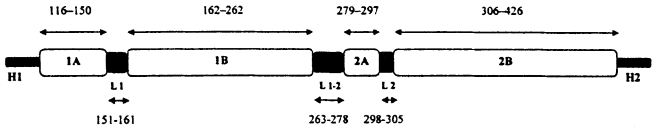
Reprint requests to: Dr. H. Scheffer, Department of Medical Genetics, University of Groningen, Antonius Deusinglaan 4, NL-9713 AW Groningen, The Netherlands. Email: h.scheffer@medgen.azg.nl

Abbreviations: *KRT14* and *KRT5*, genes encoding for keratin 14 and 5, respectively; EBS-DM, epidermolysis bullosa simplex subtype Dowling–Meara; EBS-K, epidermolysis bullosa simplex subtype Koebner; REBS, recessive epidermolysis bullosa simplex.

The major subtypes of EBS are caused by molecular defects in either the keratin 14 (*KRT14*) gene, located at 17q12–q21, or the keratin 5 (*KRT5*) gene, located at 12q13. The gene products are the epidermal keratins K14 and K5, respectively. K14 belongs to the type I keratins, or acidic keratins, and has a molecular weight of 50 kDa, whereas K5 belongs to the type II keratins, or basic keratins, and has a molecular weight of 58 kDa (Conway and Parry, 1988; Nelson and Sun, 1983). The keratins K14 and K5 are mainly expressed in basal keratinocytes, the basal cell layer of the epidermis. Together these proteins form a 10 nm keratin intermediate filament structure composed of dimeric molecules each containing a K5 and a K14 chain (Hatzfeld and Weber, 1990; Steiner, 1990; Fuchs, 1996). The keratin intermediate filament coiled coil helical-rod is subdivided into helices 1A, 1B, 2A, and 2B connected by three short nonhelical linker segments, linkers L1, L1-2, and L2, respectively (Conway and Parry, 1988) (**Fig 1**). A single mutation in *KRT14* or *KRT*5 can lead to disturbance of the filament network resulting in skin fragility and blistering. *KRT*5 and *KRT14* mutations cluster within the rod ends of 1A and 2B and in the nonhelical linker region L1-2. A summary of reported *KRT14* mutations is given in **Fig 1**.

KRT14 mutation detection on a DNA level is hampered by the presence of a highly homologous *KRT14* pseudogene. The exon and the intron sequences of the functional *KRT14* gene and the *KRT14* pseudogene are 95% and 93% identical, respectively. The *KRT14* pseudogene, however, is not being transcribed/translated because of



Number	Codon	Domain	Mutation	Phenotype	Reference
1	104	head	373delGC	EBS-WC	Rugg et al., 1994
2	116	1A	K116N	EBS-WC	Sørensen et al., 1999
3	119	1A	M119I	EBS-WC	Chen et al., 1995
4	119	1A	M119T	EBS-DM	Shemanko et al., 1998
5	120	1A	Q120R	EBS	Chen et al., 1995
6	122	1A	L122F	EBS-K	Yamanishi et al., 1994
7	123	1A	N123S	EBS-DM	Sørensen et al., 1999
8	125	1A	R125H	EBS-DM	Chen et al., 1995; Coulombe et al., 1991; Stephens et al., 1993; this study
9	125	1A	R125S	EBS-DM	Chen et al., 1995
10	125	1A	R125C	EBS-DM	Chen et al., 1995; Coulombe et al., 1991;Hachisuka et al., 1995; Stephens et al., 1993; this study
11	129	1A	Y129D	EBS-DM	Chan et al., 1996
12	143	1A	L143P	EBS-K	Sørensen et al., 1999
13	144	1A	E144A	REBS	Hovnanian et al., 1993
14			g.1842-2A>C	REBS	Jonkman et al., 1996; this study
15	204	1B	Y204X	REBS	Chan et al., 1994
16	247	1B	A247D	EBS-K	Unpublished
17	270	L1-2	V270M	EBS-WC	Rugg et al., 1993
18	272	L1-2	M272R	EBS-K	Humphries et al., 1993
19	273	L1-2	D273G	EBS-WC	Müller et al., 1998
20	274	L1-2	A274D	EBS-WC	Chen et al., 1995
21	305	L2	W305X	REBS	Corden et al., 1998
22	375	2B	E375del	EBS-WC	Chen et al., 1993
23	377	2B	I377N	EBS	Chen et al., 1995
24	384	2B	L384P	EBS-K	Bonifas et al., 1991; Hachisuka et al., 1995
25	388	2B	R388C	EBS	Chen et al., 1995
26	415	2B	Y415H	EBS-K	This study
27	419	2B	L419Q	EBS-DM	This study
28	422	2B	E422K	EBS-WC	This study

Figure 1. A schematic presentation of the keratin 14 intermediate filament helical-rod and a summary of reported keratin 14 mutations identified in patients with EBS. The numbering indicates the codon numbers according to Marchuk *et al* (1985).

the presence of some deleterious mutations, including frameshift mutations, alterations in intron/exon boundaries and disruption of the polyadenylation signal (Savtchenko *et al*, 1988). Consequently, mutation analysis in EBS has mostly been carried out on cDNA synthesized from *KRT5* and *KRT14* transcripts in mRNA isolated from skin biopsies. For *KRT5,* a genomic mutation detection system has already been described (Stephens *et al*, 1997). Here we present a method for specific amplification of genomic DNA of *KRT14* exons 1, 4 and 6 that code for the 1A, L1-2, and 2B domains containing the mutation hotspots. Our method thus obviates the need of *KRT14* cDNA synthesis from keratinocytes.

MATERIALS AND METHODS

The clinical phenotypes of the patients included in this study are summarized in **Table I**. DNA was isolated from peripheral blood by the high salt/chloroform extraction method (Miller *et al*, 1988). Prior to amplification of exons 1, 4, and 6 by the polymerase chain reaction (PCR), the genomic DNA was digested using the restriction enzyme *Taq*I, *Sph*I, or *Mse*I, respectively, that cleaves the homologous keratin 14 pseudogene sequences while leaving the *KRT14* gene sequences intact. The primer sequences, the PCR programs for amplification of the particular amplicons and the restriction enzymes used prior to PCR are given in **Table II**. PCR was carried out using 37 pmol of each primer, 500 ng digested genomic DNA, 0.6 U of rTaq DNA polymerase (Pharmacia Biotech, Piscataway, NJ), 0.2 mM dNTP in a reaction mix containing 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% wt/vol gelatin and 0.1% vol/vol Triton x-100 in a total volume of 50 μl under paraffin oil. In the case of exon 1, 10% vol/vol of dimethylsulfoxide was added to the PCR mix. After amplification, the PCR products were purified using the high pure PCR product purification kit (Boehringer Mannheim, Basel, Switzerland). Subsequently, the PCR fragments were subjected to direct sequencing using an automated DNA sequencer (ABI 377, Perkin Elmer, Norwalk, CT).

In patients with REBS, mutation analysis of *KRT14* exon 2 containing the 1B domain of K14 was carried out using a similar method as previously described (Jonkman *et al*, 1996).

When a mutation was detected its presence was confirmed by sequencing of the anti-sense strand. In those cases where the mutation abolished or created a restriction site the presence of the mutation was confirmed by restriction analysis.

RESULTS

Genomic *KRT14* mutation analysis When using the primers given in **Table II**, *Taq*I, cleaving the pseudogene sequence at the end of exon 1, *Sph*I, cleaving the pseudogene sequence at the end of intron 3, and *Mse*I, cleaving the pseudogene sequence at the start of intron 6 leave the *KRT14* sequences intact (**Fig 2**). From the few *KRT14* polymorphisms described in the literature, none actually creates a restriction site for the enzyme used to cleave the pseudogene sequence of that particular amplicon. Thus, we could amplify the *KRT14* exons 1, 4 and 6, whereas the pseudogene sequences were exempted from PCR amplification.

Table I. Clinical phenotypes and *KRT14* mutations of patients included in this study

Patient	Clinical phenotype	*KRT14* mutation	Inheritance
Patient I	EBS-DM	R125C AD[a]	*De novo*
Patient II	EBS-DM	R125C AD[a]	*De novo*
Patient III	EBS-DM	R125H AD[a]	Familial
Patient IV	EBS-DM	L419Q AD[a]	*De novo*
Patient V	EBS-K	Y415H AD[a]	*De novo*
Patient VI	EBS-WC	E422K AD[a]	Familial
Patient VII	EBS-WC	E422K AD[a]	Unknown
Patient VIII	REBS	g.1842-2A→C AR[b]	Familial
Patient IX	REBS	g.1842-2A→C AR[b]	Familial

[a]Autosomal dominant; [b]autosomal recessive.

Mutation detection In *KRT14* exon 1 of patients I and II (EBS-DM), a C to T transition at nucleotide 433 was identified (data not shown). The transition changes codon 125 for arginine (CGC) into a codon for cysteine (TGC). This mutation is designated R125C (codon numbering according to Marchuk *et al*, 1985). The mutation was confirmed by digestion with *Aci*I (data not shown). Its presence was excluded in the parents of these patients (data not shown), implying a *de novo* occurrence in both cases.

In *KRT14* exon 1 of patient III (EBS-DM), a different transition was shown in codon 125 (data not shown). In this case, a G to A transition at nucleotide position 434 was identified by which the codon 125 sequence CGC coding for arginine changes into the sequence CAC coding for histidine. This R125H mutation was also identified in the patient's mother who also had EBS-DM (data not shown).

In *KRT14* exon 6 of patient IV (EBS-DM), a T to A transversion at nucleotide position 3647 was identified (**Fig 3**). This transversion changes codon 419 for leucine (CTG) into a codon for glutamine (CAG). This novel mutation, L419Q, was confirmed by digestion with *Pst*I (data not shown). Its absence in both unaffected parents implies that it had occurred as a *de novo* event.

In patient V (EBS-K), a T to C transition in *KRT14* exon 6 at nucleotide 3634 was identified (**Fig 3**), resulting in the change of the codon 415 sequence TAC coding for tyrosine into the sequence CAC coding for histidine. This novel mutation, Y415H, was excluded in both parents. Thus, it must have occurred *de novo*.

In *KRT14* exon 6 of the unrelated patients VI and VII (EBS-WC), a G to A transition at nucleotide position 3655 was identified (**Fig 3**). Owing to this transition, codon 422 for glutamic acid (GAG) had changed into a codon for lysine (AAG). This again novel mutation, E422K, was also present in an affected sibling of patient VI. DNA from family members of patient VII was not available for analysis.

In the DNA of unrelated patients VIII and IX (REBS) identical homozygous mutations were identified (data not shown). It concerns an A to C transition at nucleotide position 1840 (g.1842-2A→C), situated at the junction of intron 1 and exon 2 of *KRT14*. This mutation is a known splice site mutation and has been described previously in three REBS patients in an apparently unrelated family (Jonkman *et al*, 1996).

Table II. Primers and PCR programs for keratin 14 hotspot mutation detection

Exon	Enzyme	Forward primer		Reverse primer		PCR program	Amplicon
1	*Taq*I	5′-cagctccatgaagggctcc-3′		5′-gagctagctggaatggtgcc-3′		A	93–626
2	*Alu*I	5′-gacaaattacctgtgccttt-3′		5′-gcccaagagtcttattcttt-3′		B	1810–2061
4	*Sph*I	5′-caggcctaaggaacaccaat-3′		5′-gagaatgccattcacaccag-3′		C	2910–3212
6	*Mse*I	5′-gaacggccacactcactaat-3′		5′-cattagatacatggtggggc-3′		C	3347–3732
A:	95°C	3′	60°C	1′	72°C	1′	1×
	94°C	1.5′	60°C	1′	72°C	1′	3×
	94°C	1.5′	58°C	1′	72°C	1′	4×
	94°C	1.5′	56°C	1′	72°C	1′	4×
	94°C	1.5′	55°C	1′	72°C	1′	18×
B:	94°C	4′					1×
	94°C	1′	52°C	1′	72°C	1′	30×
	72°C	6′					1×
	20°C	10′					1×
C:	94°C	45″	56°C	1′	72°C	45″	30×

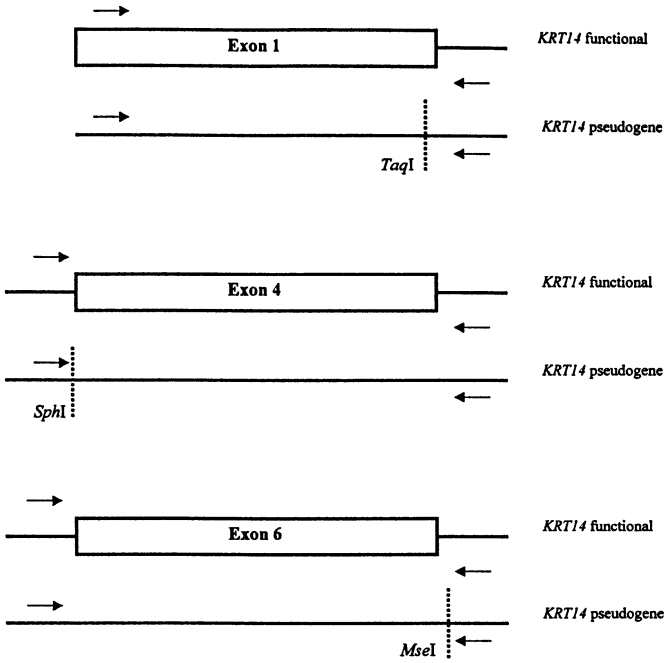


Figure 2. A schematic presentation of the position of the recognition sites of the particular restriction enzymes, cleaving the pseudogene sequences while leaving the functional *KRT14* sequences intact.

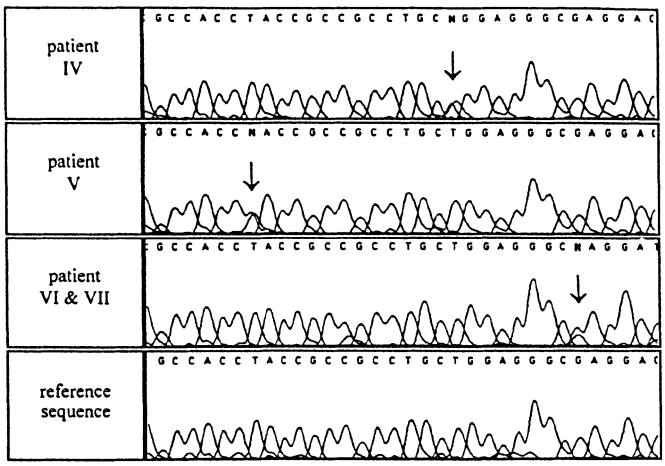


Figure 3. Novel *KRT14* exon 6 mutations. The identified base changes are indicated by arrows and are designated L419Q (patient IV, EBS-DM), Y415H (patient V, EBS-K), and E422K (patients VI and VII, EBS-WC), respectively.

In this study about 45% of the mutations occurred as a *de novo* event, 45% of the mutations are familial and of 10% of the mutations their possible occurrence as a *de novo* event is unknown. Of all *KRT14* mutations described in the literature about 25% are documented as a *de novo* event, 60% of the mutations are familial, and of approximately 15% of the mutations their occurrence as a possible *de novo* event was not documented.

DISCUSSION

The presence of a highly homologous but nontranscribed *KRT14* pseudogene has hampered genomic mutation analysis of *KRT14*. For this reason, *KRT14* mutation analysis has thus far been carried out on the transcribed *KRT14* gene, i.e., on cDNA synthesized from *KRT14* mRNA isolated from skin biopsies. The method described here makes use of restriction enzymes which cleave the *KRT14* pseudogene genomic sequences, while leaving the homologous genomic sequences of the functional gene intact. Subsequently, the mutation hotspot-containing exons of the functional *KRT14* gene are amplified, followed by direct sequencing of the PCR products. Using this approach in combination with the *KRT5* mutation analysis (Stephens *et al*, 1997), we could detect all mutations in 16 patients with EBS. Consequently, EBS mutation analysis of the *KRT14* gene might initially be limited to the above-mentioned amplicons.

A recently published alternative (Sørensen *et al*, 1999) based on the principle of mismatching with the *KRT14* pseudogene sequences, makes use of primers specific for the amplification of the functional *KRT14* gene sequences. A drawback of this method may be that no optimal specificity of the amplification of the functional gene can be obtained, as the mismatches are not always located at the 3′ end of the primers.

Using our novel method, we identified six different *KRT14* mutations in 16 patients. In the two REBS patients the mutation g.1842-2A→C, a known splice site mutation (Jonkman *et al*, 1996), is identified. Haplotyping (results not shown) indicated that this mutation is most likely a founder mutation in the north-eastern part of the Netherlands.

In three EBS-DM patients, codon 125, situated in domain 1A of the K14 protein, appeared to be mutated. The mutations involved, R125H and R125C, have previously been described in association with EBS-DM (Coulombe *et al*, 1991; Stephens *et al*, 1993; Chen *et al*, 1995; Hachisuka *et al*, 1995).

The three novel mutations Y415H (EBS-K), L419Q (EBS-DM), and E422K (EBS-WC), identified in four unrelated EBS patients, occur in highly conserved residues at the end of the K14 rod domain 2B (Fuchs, 1996). The conservation of these residues is assumed to reflect functional importance. A number of studies,

including cross-linking experiments (Steinert *et al*, 1993), cell-based assays (Letai *et al*, 1992), and structural analysis of synthetic peptides (Geisler *et al*, 1993) have indicated that this region, within the helix termination motif, is implicated in end-to-end interactions in keratin filament formation. Whereas mutations in *KRT*5 helix termination motifs have already been described (Lane *et al*, 1992; Irvine *et al*, 1997; Müller *et al*, 1999) no such mutations had as yet been reported for the helix termination motifs of *KRT14*.

It is remarkable that not all of the identified novel mutations in the phylogenetically highly conserved helix termination motif cause the severe EBS-DM phenotype (L419Q), but also result in the milder EBS-K (Y415H), and EBS-WC (E422K) phenotypes. The phenotype is constant between affected members of the same family. The clinical phenotype does not correlate with the position of the changed residue in the heptad repeat, as all changes occur at either the a or the d positions of the heptad repeat, that are both oriented inwards in the helix coiled-coil. It can be questioned, however, whether the helix termination motif has a true coiled-coil conformation. The mild Koebner phenotype of the patient carrying the Y415H mutation may reflect the fact that both the tyrosine (Y) as well as the histidine (H) residues occur frequently at the heptad repeat d position in the keratin gene family (Conway and Parry, 1988).

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